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Carcinogenesis Assay of Subfractions of Cigarette Smoke Condensate Prepared by Solvent-Solvent Separation of the Neutral Fraction [1,2]

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SUMMARY—Carcinogenesis assay was conducted on subfractions of the neutral fraction (NF) of cigarette smoke condensate, subfractions that were prepared by solvent partition. Of the 2 major subfractions, the "methanol-insoluble" neutrals (MIN) were much more active than the "methanol-soluble" neutrals (MSN). Distribution of MSN between nitromethane and carbon disulfide yielded 2 active fractions. Poor dose-response effects suggest that extraneous materials may reduce the activity of MSN. Analysis of the recovery of MSN is difficult, but apparently there were significant losses during separation. On countercurrent distribution (CCD), MIN yielded 3 active and 2 inactive subfractions. Recovery of the activity of MIN in the subfractions was excellent in comparison with an earlier separation with silicic acid chromatography. Recoveries might be improved if CCD is applied to the NF before the more rigorous chromatographic separations.—J Natl Cancer Inst 49: 477–483, 1972.

IN AN earlier study, 4 of the subfractions prepared by silicic acid chromatography of the neutral fraction (NF) of cigarette smoke condensate (CSC) induced skin tumors in mice previously painted with 125 μg of 7,12-dimethylbenz[*a*]anthracene (DMBA) (*1*). With this procedure, tumor-promoting agents as well as complete carcinogens were detected. All the subfractions were recombined in proportion to their yield, to provide a reconstituted sample that would have been identical with NF if no changes had occurred during the separation procedure. The biological activity of this reconstituted material, however, was substantially less than that of NF, suggesting that important amounts of the active materials were lost during chromatography. The current study was undertaken to examine solvent partitioning alone as means of fractionating NF, with the hope that these losses could be avoided.

MATERIALS AND METHODS

Details of the preparation of the various fractions will be described elsewhere (*2*). Briefly, 1-kg

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batches of CSC were fractionated as before to provide the NF (*3*). Each batch of NF was then distributed in a 2-phase system containing cyclohexane and aqueous methanol. From the cyclohexane phase, "methanol-insoluble" neutrals (MIN), an aliquot was removed for control bioassay and the rest was condensed. [MIN is thus identical with the cyclohexane layer of our former separation scheme (*3*) *before* its partition with nitromethane to yield F13 and F14.] The aqueous methanol layer, "methanol-soluble" neutrals (MSN), is identical with the aqueous methanol layer, F12, of that scheme. The MIN was subjected to a 200-tube counter-current distribution (CCD) in a solvent system consisting of cyclohexane and nitromethane. Five subfractions were obtained; 40% of each was removed and pooled to provide reconstituted MIN. From the original MSN an aliquot was removed for control bioassay and the remainder was condensed and partitioned between nitromethane and carbon disulfide in the funnel. Two subfractions were obtained: the MSN-soluble in nitromethane; and the MSN-soluble in carbon disulfide. From each 40% was pooled to provide a reconstituted MSN.

The fractions and the reconstituted MIN and MSN were freed of solvent, shipped under dry ice from Philadelphia to Buffalo, and kept in a cold room until used. Each fraction was diluted so that its final concentration was equivalent to a specific concentration of CSC. That is, if the fraction were obtained from CSC with a yield of 1%, it would be diluted to 0.60% to be equivalent to a 60% solution of CSC. If another fraction were recovered with a yield of 2%, it would correspondingly be diluted to 1.20%, to be equivalent to 60% CSC (*see* table 1 for the average concentrations used).

The biological assays were generally conducted as in our earlier studies (*1*, *4*). The mice were pretreated at 11 weeks of age with a single, initiating dose of 125 μg DMBA dissolved in 0.25 ml acetone. The concentration of the DMBA solution was confirmed by examination of its ultraviolet absorbance at 363 nm. After 3 weeks, the animals were painted 5 times weekly with the promoting stimuli, consisting of 0.20 ml of acetone solutions of the test materials delivered with a 0.2-ml Biopette (Carworth Lab Cages, New York, N.Y.). Croton resin (C.R.) was prepared from croton oil by a procedure suggested by Van Duuren (personal communication). The yield of resin was such that 0.0025% C.R. would be obtained from 0.1% croton oil. The promoting activity of 0.0025% C.R. was approximately equal to that of 0.05% croton oil in our laboratory. The experiment was terminated after 64 weeks.

RESULTS AND DISCUSSION

At 1 year of age (38 weeks of promoting stimulus), 91% of the mice were alive. Survival did not vary significantly among the groups at that time. At the end of the experiment, when the mice were 78 weeks old, 38% were alive (table 1).

Controls

No tumors appeared in the mice after treatment with acetone only. Among 50 mice treated with DMBA followed by acetone, 4 developed 1 tumor each. All of these tumors appeared within 52 weeks. Although the incidence of 4 tumors in the DMBA + acetone group was higher than usual, it was not significantly above our laboratory experience of the past few years, in which approximately 3.5% of animals treated with DMBA followed by acetone developed tumors at 52 weeks, and 5.6% at 64 weeks. The incidence of tumors among the mice painted with DMBA followed by C.R. was within the range we had observed in other experiments.

Active Subfractions

To assess the relative merits of CCD and column chromatography, it is necessary to compare the various active subfractions obtained with each method. The fractions and subfractions were tested at concentrations proportional to their concentrations in the original CSC from which they were obtained. The MIN and MSN test solutions were the equivalent of 30 or 15% CSC. The concentrations of the subfraction test solutions were relatively twice as high, *i.e.*, the equivalent of 60 or 30% CSC. Accordingly, any subfraction that proved active may have contributed importantly to the activity of the crude material. Conversely, a subfraction that was inactive might, nevertheless, contain carcinogens or tumor promoters, but in

TABLE 1.—Development of tumors in mice treated with NF subfractions

Fraction No.	Treatment	Relative concentration (equivalent % CSC)	Mg/dose (average)	Number of mice*			Maximum No. of skin tumors
				Survivors	With tumors	With skin cancers	
		Controls					
—	Acetone	—	—	17	0	0	0
—	DMBA + acetone	—	—	23	4	0	4
—	" + 0.0025% C.R.	—	—	9	37†	16	
		MeOH-insoluble neutrals					
32	DMBA + unfractionated	60	27.	21	32†	14	61
33	" + reconstituted	60	27.	9	29†	3	43
34	" + subfraction 0–20	60	0.4	20	4	1	6
35	" + " 21–45	60	1.0	26	7	4	8
36	" + " 46–140	60	4.0	8	36†	15	63
37	" + " 141–165	60	3.4	20	7	1	9
38	" + " 166–199	60	17.	14	4	0	3‡
32	" + unfractionated	30	14.	15	27†	9	42
34	" + subfraction 0–20	30	0.2	16	5	0	4‡
35	" + " 21–45	30	0.5	22	8†	3	8
36	" + " 46–140	30	2.0	14	26†	3	38
37	" + " 141–165	30	1.7	26	11†	3	15
38	" + " 166–199	30	8.4	21	5	0	4
32	" + unfractionated	15	6.8	17	18†	9	22
		MeOH-soluble neutrals					
12	DMBA + unfractionated	60	5.6	18	12†	2	16
40	" + reconstituted	60	5.6	21	9†	3	7‡
41	" + nitromethane-soluble	60	4.5	25	11†	5	15
42	" + CS_2-soluble	60	1.6	23	8†	3	11
12	" + unfractionated	30	2.8	24	6	1	5‡
41	" + nitromethane-soluble	30	2.3	19	2	0	5
42	" + CS_2-soluble	30	0.8	20	7	3	7
12	" + unfractionated	15	1.4	26	9†	5	7‡

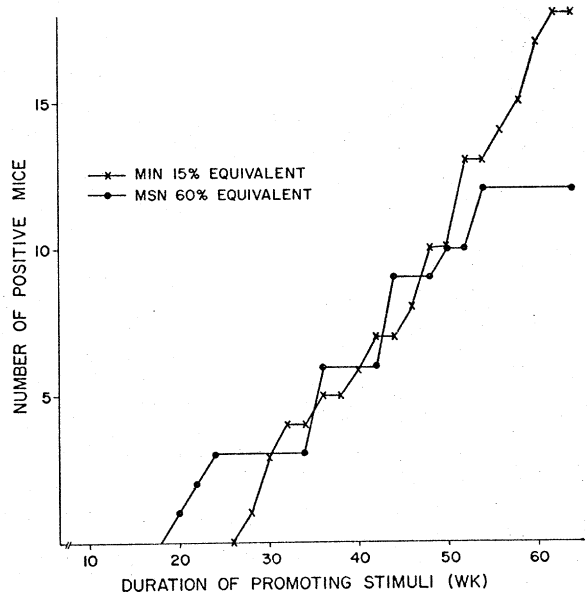
*After 64 weeks of test (78 weeks of age); 50 mice per group.

†$P<0.01$ in comparison with pooled negative controls from several experiments.

‡In these mice, one or more tumors regressed after appearing for at least 3 weeks. Mice were counted as positive, but the tumors were dropped from the tabulation.

concentrations too small for them to be important contributors to the activity of the unfractionated starting materials. Many such trace carcinogens have been identified in CSC (*5*, *6*). The major part of the activity of the crude NF appeared in the MIN. Apparently, the MIN is about 4 times as potent as the MSN (text-fig. 1). Fractionation of MSN from carbon disulfide and nitromethane gave 2 active subfractions (table 1). Both subfractions contributed importantly to the activity of the original MSN. Even so, we cannot assume the presence of 2 different active constituents, because many individual compounds could have been distributed so that significant amounts appeared in both subfractions.

Two inactive and three active subfractions were obtained by CCD of MIN (table 1). Each of the 3 active subfractions could contribute importantly to the activity of the unfractionated MIN. In this case, at least 2 active constituents must be present. The distribution of benzo[*a*]pyrene (BP) among the subfractions indicates the efficiency of the large-



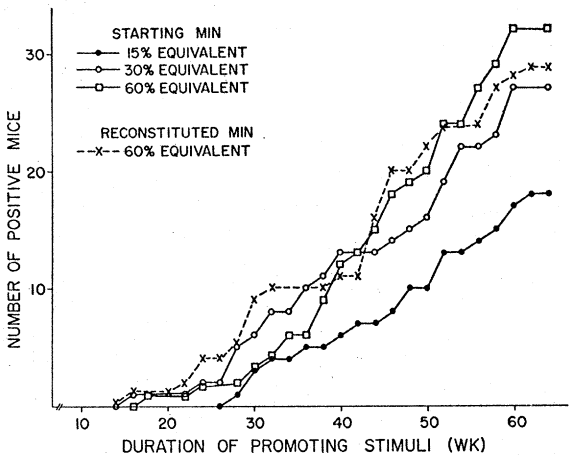
TEXT-FIGURE 1.—Comparison of MIN with MSN. Each group consisted of 50 mice treated once with 125 μg DMBA. The test solutions were applied 5 times weekly, beginning 3 weeks after the initiating stimulus.

scale CCD separation. Nearly all the BP appeared in tubes 46–140, according to the prediction based on the measured distribution coefficient (K) of BP in the solvent pair (*2*). Under the circumstances, we assumed that other constituents of MIN were also distributed according to their respective K's. A single compound could not be distributed in important quantities among all 3 fractions. If its K equaled that of BP, it would appear only in tubes 46–140, as did BP. If its K were smaller, it could not appear in tubes 141–165; if its K were greater, it could not appear in tubes 21–45. Accordingly, the results required at least 2 constituents, with different K's, that contribute importantly to the activity of the MIN. Possibly, all 3 of the active subfractions of MIN owe their activity to a series of compounds with similar structure. Hoffmann and Wynder demonstrated that a mixture of 17 polynuclear aromatic hydrocarbons (PAH) may account for some of the biological activity of CSC (*7*). It would be interesting to learn whether these compounds have sufficiently diverse K's to account for our observations.

These data, together with data obtained earlier, indicate a minimum of 4 distinctly different fractions of CSC that can be expected to contribute importantly to its activity: the non-steam-distillable, ether-soluble weak acids (*8*); the methanol-eluted, MSN subfraction from the silicic acid column (MMw); and at least 2 subfractions of MIN. CSC contains many constituents that are known or believed to be skin carcinogens, tumor initiators, or tumor promoters (*5*, *6*, *9*), but most of them are present in very small concentrations and their individual significance has not been demonstrated.

Recovery and Usefulness of the Separation Procedure

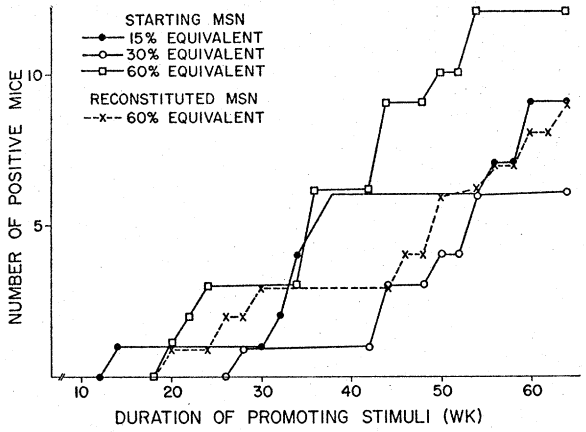
The recoveries and efficiencies of separation obtained can be compared with those we reported in 1969 (*4*) and 1970 (*1*). In 1969, we found the 3 neutral subfractions that were separated by solvent-solvent techniques were all active. One of these was MSN; and the other 2, combined, would comprise the MIN of this study. Recovery of activity in the fractionation of MIN appeared good (text-fig. 2). In 1970, we obtained 4 active neutral subfractions



TEXT-FIGURE 2.—Tumor induction by MIN before fractionation, and by reconstituted MIN prepared by recombination of proportionate amounts of all subfractions.

prepared by silicic acid chromatography followed by solvent partitioning. One of these, the methanol-eluate material soluble in 90% methanol (MMw), was more polar, resembling MSN. The other 3 probably contained the same active constituents as MIN in the present study.

The MSN of this study produced the same poorly defined dose-response effects as observed earlier (*4*). In 1969, more tumors were produced by the dilute MSN than by the more concentrated solution, which contained twice as much material. In the present study, the most dilute MSN solution produced cancer in more mice than did the most concentrated MSN solution, which was 4 times as concentrated (table 1). Likewise, the number of mice with tumors was about equal in the 2 groups during the first 42 weeks (text-fig. 3). It is noteworthy, however, that the MMw described in our 1970 publications produced many more tumors in 1970 than did MSN in 1969 or 1971 (*1*, *10*). Possibly, the reverse dose-response effects noted with the MSN of both experiments 1 and 3 are caused by the extraneous material of this fraction interfering with tumor induction. The MMw from the column contained only one-third to half as much material as the MSN. The apparent difference in activity thus may be due to the removal of interfering extraneous material during column separation. Extraneous material could reduce the tumor yield by interfering with penetration into the skin, by indirect effects on the host, or by more



TEXT-FIGURE 3.—Tumor production by MSN before fractionation and by the reconstituted MSN.

specific interference in metabolic processes associated with carcinogenesis (*11–13*). Whether any of these mechanisms is involved in the present results cannot be ascertained until the constituents of MSN are identified.

Because of the atypical dose-response effects, the recovery of MSN is difficult to evaluate. Probably some of the active material from MSN was lost during its subsequent fractionation. Because of the difficulties in interpretation of the dose-response effects, however, the extent to which losses occurred cannot be ascertained.

Distribution of MSN between nitromethane and carbon disulfide did not give a preponderance of active material in either of the 2 subfractions (table 1, fractions 41 and 42). Three-fourths of the total mass of MSN appeared in the nitromethane-soluble subfraction. Thus, if the activity was distributed between the 2 solvents about equally, the concentration of the active material was about 3 times as high in the carbon disulfide subfraction. Assuming a single component or a group of similar components with nearly equal partition coefficients, CCD procedures with these solvents should be useful in further removal of the active compounds from the bulk of the extraneous constituents. CCD might also separate the active constituents, should more than one exist. The efficacy of such a procedure, however, depends on the magnitude of the losses incurred in separation. The present study indicates that such losses may be important. Other methods of separation might prove more useful.

Countercurrent distribution was a potentially useful tool to separate the less polar substituents. Recovery of activity in the reconstituted MIN was excellent, in marked contrast to our earlier experiment in which the total neutrals were separated on a silicic acid column [text-fig. 1, this paper, and text-fig. 3 (*1*)]. During most of the experiment, as many or more tumors were produced by the reconstituted material as by the unfractionated NF from which it was derived. With the earlier silicic acid chromatography, the reconstituted NF equivalent to 60% CSC produced fewer tumors than the starting NF equivalent to only 15% CSC.

Five major subfractions of MIN were collected; three of them were active. These may compare to 3 active fractions from the silicic acid column. The 3 active subfractions of MIN obtained by CCD comprised 30% of the total mass. With silicic acid chromatography, the 3 active fractions that may be comparable contained only 18% of the mass. At first inspection, it appeared that silicic acid chromatography was a more efficient method for isolation of the active ingredients of MIN from the inactive constituents. Two considerations argue against this conclusion. First, substantially better recovery of active material resulted from the CCD procedure [text-fig. 1, this paper, and text-fig. 3, (*1*)]. Second, the bulk of the inactive mass appeared in only 1 tail of the CCD separation. It should be possible to arrange a modified CCD scheme that would be more efficient as to time in separation of nearly all of the active materials from most of the inactive constituents. The CCD product might then be subjected to other separation systems. This sequence of operations could provide the advantage of use of more stringent conditions when the volumes of material were reduced and the problems of large-scale separation could be controlled more conveniently.

Importance of Promoters in CSC

With mice pretreated with DMBA, both tumor promoters and complete carcinogens will be active. We reported earlier that the ether-soluble, non-steam-distillable weak acids are probably tumor promoters rather than complete carcinogens. In an experiment still under way, we are determining

whether the various fractions eluted from the silicic acid column have tumor-initiating activity as well as tumor-promoting activity. Preliminary data indicated that MMw has very little if any tumor-initiating activity when applied to mice subsequently treated with croton oil. If the final results confirm this early finding, we may consider that MMw, like the weak acids, is probably a promoter only, and not a complete carcinogen. The MSN of the present study probably contains the same active materials as MMw. As a working hypothesis, then, one may assume that MSN is a tumor promoter and not a complete carcinogen.

Several laboratories have tested various fractions of CSC as complete mouse skin carcinogens (*14–16*). Often, there have been noticeable losses in activity as the fractionation has progressed, despite the fact that recovery of polycyclic hydrocarbons by the same general procedures is excellent. Our data indicate that at least 2 fractions, the non-steam-distillable weak acids and the polar NF, which may contribute importantly to the tumor-promoting activity of CSC are tumor promoters only. It may thus be questioned whether losses of complete carcinogenic activity during fractionation of CSC are due to separation of these tumor promoters from other fractions that possess initiating activity. Several workers have suggested that cigarette smoke induces a typical 2-stage system of carcinogenesis (*17–20*). Van Duuren *et al.* (*21*) and Roe *et al.* (*17*) have provided evidence to support this concept. Hoffmann and Wynder showed that when a mixture of PAH was added to CSC so as to double or triple their normal concentration, the final tumorigenic activity was increased accordingly (*7*).

The last observation suggests that CSC acts as a promoter containing a limited amount of initiating substances. To determine the precise nature of these interactions will require the recombination of individual agents from CSC so that their individual contributions to the whole may be estimated. The present study demonstrates that numerous agents may contribute importantly to the behavior of CSC in an assay system that detects both complete carcinogens and tumor promoters. Whether the fractions are important for expression of the complete carcinogenic activity of CSC must await further study.

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